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# Differential Synthesis of Bacteriophage-specific Proteins in MS2-infected *Escherichia coli* treated with Actinomycin

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*Escherichia coli* pretreated with EDTA and actinomycin remained susceptible to infection with MS2, although the efficiency of infection and burst size were reduced markedly at high concentrations of actinomycin. At an optimum concentration of actinomycin the synthesis of protein and RNA was largely dependent on bacteriophage infection. Phage-specific protein synthesis was followed by measuring incorporation into protein of histidine, an amino acid not present in the phage coat protein, and arginine or lysine, which are present in the coat protein. The maximal rate of histidine incorporation into the proteins of the infected cell was reached about six minutes earlier than the maximal rate of arginine incorporation, and histidine incorporation levelled off sooner. The total number of coat protein molecules made during the replicative cycle exceeded the number of histidine residues incorporated by a factor of three to five. Protein synthesis was shown to be dependent on the formation of RNA by measuring amino acid incorporation into protein under a variety of conditions where little or no viral RNA was made. Under every such condition the incorporation of both histidine and arginine (or lysine) was inhibited. It is concluded that the protein synthesis observed occurs on newly formed RNA templates, and that the translation of different cistrons of progeny RNA is regulated with regard to time and rate.

## 1. Introduction

Infection of susceptible cells by an RNA virus leads to the production of new enzyme(s) and structural proteins synthesized under the direction of viral nucleic acid. Such systems offer the possibility of studying the regulation of protein synthesis at the level of messenger RNA function, since the viral RNA serves as a template for phage-specific protein synthesis (Nathans, Notani, Schwartz & Zinder, 1962), and its template function in the infected cell is presumably regulated during virus development (for example, see Lodish, Cooper & Zinder, 1964).

In order to use virus-infected cells to investigate regulation of protein synthesis, it would be useful to reduce or eliminate host protein synthesis under conditions which allow virus replication to proceed. Although this has been accomplished for poliovirus by infecting sensitive animal cells in the presence of guanidine and actinomycin (Summers, Maizel & Darnell, 1965), the use of actinomycin has so far led to only partial success in the case of bacteria infected with an RNA bacteriophage (Haywood & Sinsheimer, 1965). We report here that actinomycin-treated *E. coli* (sensitized to actinomycin by the method of Leive (1965)) are susceptible to infection by an RNA phage, though with lowered efficiency and decreased burst size. At an optimum actino-

mycin concentration the synthesis of RNA and protein of the host is blocked sufficiently to allow detection of phage-specific macromolecules. Since the coat protein of coliphage MS2 contains no histidine (Naughton, personal communication), the synthesis of phage-specific non-coat protein can be directly compared with the formation of viral RNA, coat protein and intact phage particles. The results to be presented indicate that the synthesis of virus-specific proteins is regulated during the replicative cycle, both with regard to the number of molecules of different proteins formed and with regard to the time at which synthesis occurs.

A summary of part of this work has already appeared (Oeschger & Nathans, 1966).

## 2. Materials and Methods

*Bacteriophage MS2 and its host* E. coli *C3000* were obtained from R. L. Sinsheimer. Multiple amino acid auxotrophs of C3000 were isolated after ultraviolet irradiation or treatment with a chemical mutagen. (We are grateful to Y. Shimura for providing some of these mutants.) An arginine$^-$lysine$^-$histidine$^-$ strain is designated C3000–37 and a uracil-requiring mutant derived from it as C3000–38. Temperature-sensitive mutants of MS2 were isolated by C. Caplan and J. Fuller from a phage stock treated with nitrous acid (Zinder & Cooper, 1964) by plating at 34 and 42°C. Mutant stocks were prepared by single-cycle growth at low temperature. The stock used had approximately one revertant per 1000 mutant phages. Phage assays were carried out as described by Cooper & Zinder (1962) for f2, and centrifugation of phage in CsCl was done as previously described (Shimura, Moses & Nathans, 1965).

Host bacteria were grown in the TPG medium described by Davis & Sinsheimer (1963) with slight modification. It contained the following ingredients: 0·11 M-KCl, 8·0 mM-NaCl, 0·05 M-$NH_4Cl$, 0·1 M-Tris base, 0·16 mM-$Na_2SO_4$, 1·0 mM-$KH_2PO_4$, 0·073 M-sodium pyruvate, 0·1 mM-$CaCl_2$, 1·0 mM-$MgCl_2$, 2 $\mu\mu$M-$FeCl_3$, 10 mg thiamine/l. and 2 g glucose/l. Amino acid supplements were added as required, and the pH was adjusted to 7·4 with HCl.

Actinomycin D was a gift of Merck, Sharpe & Dohme, Inc. $^{14}$C-labeled amino acids were purchased from New England Nuclear Corp., Boston, Mass., and [$^3$H]uridine from Nuclear Chicago Corp., Chicago, Ill.

*Incorporation of radioactive precursors* into RNA or protein was measured by suitably washing a portion of culture on Whatman 3 MM filter paper discs previously spotted with non-radioactive precursor. In the case of protein, the discs were washed and counted as described earlier (Nathans, 1965). In the case of RNA, the discs were washed with ice-cold 5% trichloroacetic acid 5 times, alcohol–ether (1 : 1, v/v) twice, and ether twice.

*Treatment of* E. coli *with actinomycin* was carried out as follows: log-phase cells were grown in modified TPG medium to a concentration of about $8 \times 10^8$ cells/ml., centrifuged at 20°C and washed once with 0·12 M-Tris–HCl (pH 8·0). The pellet was resuspended in the same buffer at 1/10 the original volume of culture and the cell suspension kept at 4°C for 1/2 to 2 hr. The chilled cells were then mixed with an equal vol. EDTA (0·4 mM)–Tris–HCl (0·12 M, pH 8·0), prewarmed to 41°C. After 3 min at 41°C, the cells were transferred to 10 vol. of modified TPG medium prewarmed to 37°C, containing actinomycin, required amino acids, and twice the usual concentration of $Ca^{2+}$, $Mg^{2+}$ and $Fe^{3+}$ salts. After shaking for 5 min at 37°C, the cells were ready for infection with MS2.

## 3. Results

### (a) *Effect of actinomycin on RNA and protein synthesis of* E. coli *C3000*

As shown by Leive (1965), sequential treatment of *E. coli* with EDTA and actinomycin effectively shuts off RNA and protein synthesis. With strain C3000 and the condition given in Materials and Methods (actinomycin 5·5 $\mu$g/ml.), RNA and protein synthesis were nearly completely shut off for 70 to 80 minutes (see control curves of Fig. 2). In a typical experiment, histidine incorporation was reduced to about 0·001

mμ-mole/hr per $10^8$ cells; arginine, to 0·01 mμmole; and uridine, to 0·005 mμmole. At lower concentrations of actinomycin (2 to 4 μg/ml.) the inhibition of protein synthesis was less, and at higher concentrations (8 to 20 μg/ml.) complete shut-off was obtained, but the efficiency of phage infection was decreased (see below). The concentration of actinomycin which gave nearly complete inhibition of host protein and RNA synthesis, but allowed adequate phage infection, was generally 5·5 to 6·5 μg/ml. Even within this narrow range, occasional experiments were unsatisfactory due to inadequate inhibition of host protein synthesis or to low infective centres. Presumably this was due to variability in the sensitivity of different batches of cells to actinomycin.

(b) *Effect of actinomycin on infective centers and burst size*

Figure 1 shows the effect of actinomycin concentration on the formation of infective centers and on average burst size. At a concentration of 16 μg/ml., infective centers have decreased to 13% of the control value and burst size to 0·7%. In several experiments with 5·5 to 6·0 μg/ml. of actinomycin, the concentration range used in subsequent experiments, the average value of both the infective centers and the burst size was 20% of the control. The cause of this fall in efficiency of infection and in yield of infective phage in the presence of actinomycin is not known.

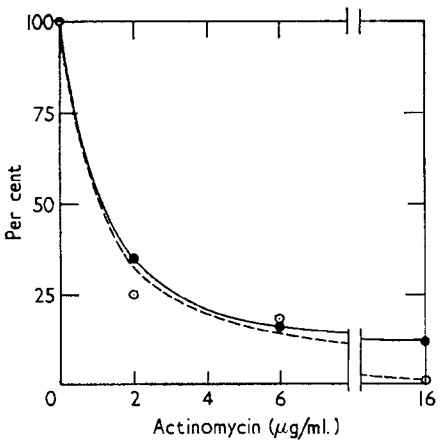


FIG. 1. The effect of actinomycin on infective centers and burst size.

*E. coli* C3000 was prepared and treated with actinomycin as described in Materials and Methods. After exposure to actinomycin for 5 min, MS2 was added at an input multiplicity of 10; 5 min later antiserum was added, each culture diluted with medium containing the appropriate actinomycin concentration, and infective centers (●) and burst size at 90 min (⊙) determined.

(c) *Protein and RNA synthesis in infected cells*

After infection of actinomycin-treated cells with MS2, protein and RNA synthesis is markedly stimulated (Fig. 2). As shown in the Figure, under the conditions given, nearly all protein and RNA synthesis was phage-dependent for the first 70 minutes after infection. In a typical experiment, the incorporation into protein of histidine, an amino acid missing from phage coat, reached a maximum rate by about 17 minutes and began to reach a plateau at 30 minutes (Fig. 3). In contrast, rapid rise in incorporation of arginine, which is present in phage coat, starts at about 23 minutes after infection

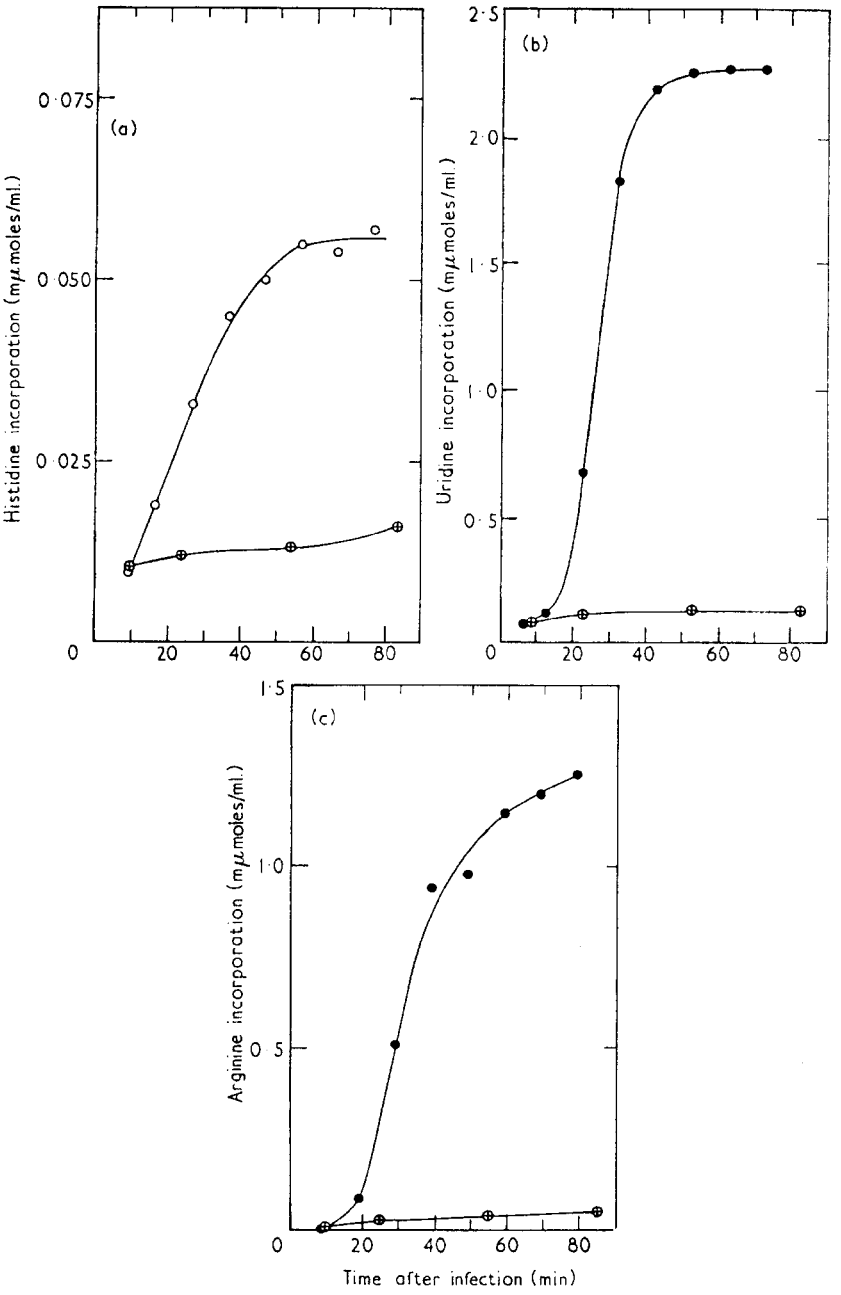


FIG. 2. Protein and RNA synthesis in actinomycin-treated cells: infected *versus* uninfected.

Strain C3000–37 was prepared in 5·5 μg actinomycin/ml. as described in Materials and Methods, in medium containing $2 \times 10^{-6}$ M-histidine, $9 \times 10^{-6}$ M-arginine, $1{\cdot}5 \times 10^{-5}$ M-lysine and $2 \times 10^{-5}$ M-uridine. One-half of the culture was infected with MS2 at a multiplicity of 20. After 5 min at 37°C, 1-ml. portions were distributed to tubes containing either [$^{14}$C]histidine (1 μc, 240 mc/m-mole), [$^{14}$C]arginine (1 μc, 240 mc/m-mole) or [$^{3}$H]uridine (10 μc, 2 c/m-mole). The total concentrations of precursors were identical in all tubes. At various times, 0·1-ml. samples were removed to filter paper discs pretreated with non-radioactive precursor, and radioactive protein or RNA determined as described in Materials and Methods. (⊕) Uninfected cultures; (●) or (○) infected cultures.

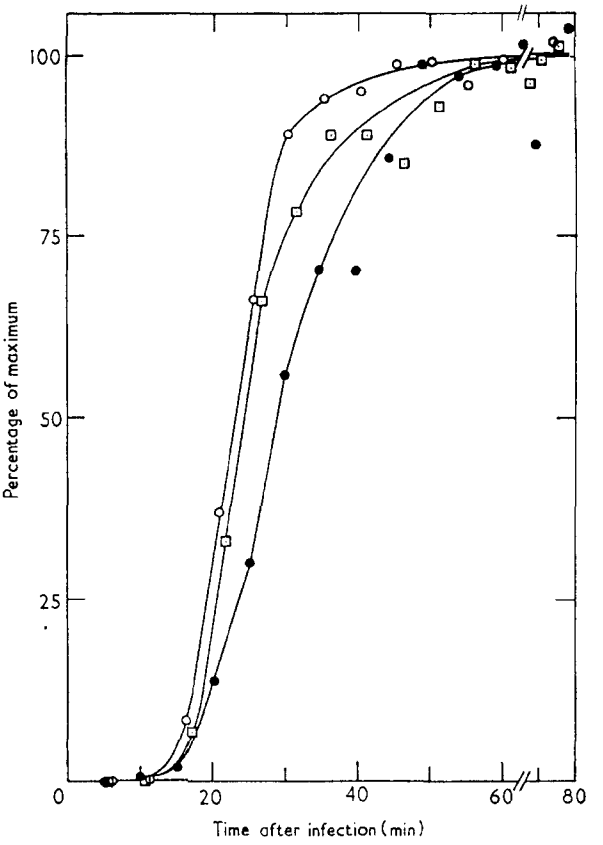


FIG. 3. Kinetics of histidine, arginine and uridine incorporation after infection.

The procedure was the same as that given in the legend of Fig. 2. Final cts/min incorporated per 0·1 ml. were as follows: histidine 520, arginine 4500, uridine 6000 in infected cells and 102, 220 and 305, respectively, in uninfected cells. For each time point the value for uninfected cells was subtracted. (○) Histidine; (●) arginine; (⊡) uridine.

and continues for a longer time than the incorporation of histidine (Fig. 3). The kinetics of incorporation of other amino acids present in phage coat protein (lysine, valine, isoleucine) was similar to that of arginine. In the same experiment RNA synthesis paralleled the incorporation of histidine, though in other experiments the duration of RNA synthesis was longer (see Fig. 7(a)). The formation of infectious phage, which corresponded temporally to phage production in *nonactinomycin* treated cells, lagged about 10 to 15 minutes behind the synthesis of arginine-containing protein (Fig. 4). That the [$^{14}$C]arginine-containing protein made after infection is mainly phage coat protein has been shown by co-chromatography of about 85% of the total labelled protein with coat protein on Sephadex G200 in the presence of guanidine (for method, see Nathans, 1965). Also, following sonication of arginine-labeled cells, about 70% of the labeled protein released (about 70% of the total) is present in phage particles, isolated by centrifugation in CsCl.

The actual amounts of the various labeled precursors finally incorporated in several experiments are shown in Table 1. As seen by a comparison of the number of subunits

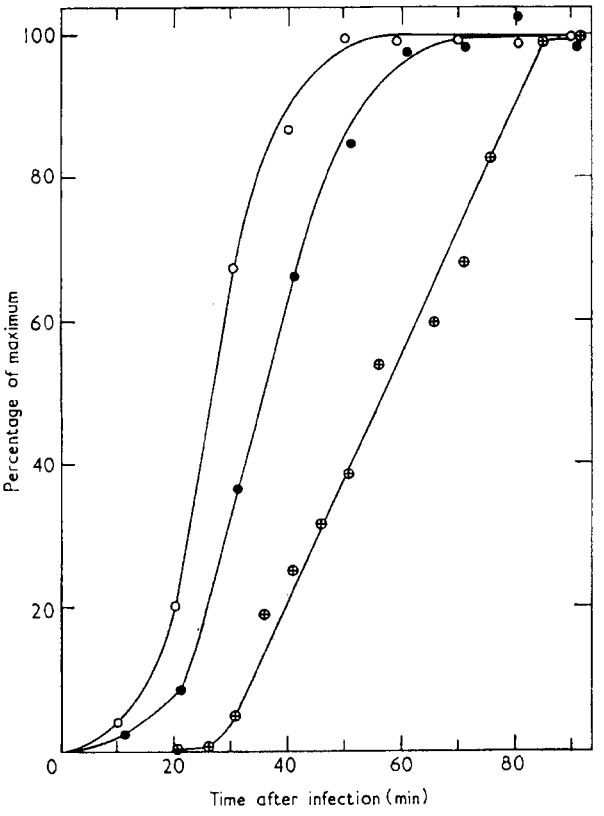


FIG. 4. Kinetics of phage formation compared with histidine and arginine incorporation.

The procedure is given in the legend of Fig. 2. For each time point the cts/min incorporated by uninfected cells was subtracted. (○) Histidine; (●) arginine; (⊕) bacteriophage.

TABLE 1

*Protein and RNA synthesis*

| Exp. | I.C./ml.† ($\times 10^{-7}$) | Burst Size | Incorporation mμmoles/ml. | | | Incorporation molecules/I.C. ($\times 10^{-6}$) | | | Equivalents/I.C. Subunits of coat protein ($\times 10^{-6}$) | Equivalents/I.C. φRNA‡ ($\times 10^{-4}$) |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | His | Arg | Uridine | His | Arg | Uridine | | |
| 1 | 2·8 | 570 | 0·036 | 0·50 | 3·1 | 7·7 | 110 | 660 | 24 | 8·3 |
| 2 | 4·7 | 530 | 0·032 | 0·73 | 6·6 | 4·1 | 90 | 840 | 19 | 11 |
| 3 | 2·5 | 48 | 0·024 | 0·45 | 4·0 | 5·7 | 110 | 970 | 24 | 12 |
| 4 | 2·9 | — | 0·004 | 0·084 | 2·1 | 0·83 | 17 | 430 | 3·7 | 5·9 |

† Infective centers/ml.
‡ Bacteriophage RNA.

of coat protein produced and the number of histidine molecules incorporated into protein, the ratio of coat protein subunits to histidine varied from 3·2 to 4·6. Therefore, even under the limiting assumption of one histidine residue per protein molecule, the infected cell makes about three to five times fewer histidine-containing protein molecules than of coat protein molecules. Also shown in Table 1 is the number of phage equivalents of RNA, which was generally about five to ten times the phage equivalents of coat protein (assuming 180 subunits per phage). After sonication of uridine-labeled cells with release of all the RNA counts, only about 10% of the labeled RNA banded in CsCl with phage particles; most of the RNA sedimented to the bottom of the tube.

### (d) *Reading of input* versus *progeny RNA*

Four types of experiments were done to determine whether the protein synthesis observed in the presence of actinomycin was due to reading of input RNA or newly formed RNA. In the first experiment the effect of the uracil analog 5-fluorouracil on phage-specific protein and RNA synthesis was determined. Treatment of actinomycin-inhibited cells with FU† five minutes before infection with MS2 resulted in marked inhibition of incorporation of histidine, arginine–lysine and adenosine (Fig. 5). (In other experiments of this type no detectable protein or RNA synthesis occurred in the presence of FU.) When FU was added later (five minutes after infection), phage-dependent protein and RNA synthesis was inhibited less, but both histidine incorporation and arginine–lysine incorporation were reduced proportionately (Fig. 5). The fact that FU had little effect on the formation of infective centers measured after diluting out FU 12 minutes post-infection (see legend of Fig. 5), indicates that the inhibition of protein and RNA synthesis was not due to failure of phage RNA to enter the cell. Since FU should not affect the expression of input RNA, but does cause reduced RNA synthesis, the fall in histidine and arginine–lysine incorporation leads to the conclusion that the synthesis of both the histidine-containing protein and coat protein are dependent on new RNA molecules.

In the second experiment, actinomycin-treated cells were infected with MS2 in which 80% of the uracil was replaced with FU (Shimura *et al.*, 1965). (We are grateful to Y. Shimura for providing this phage preparation.) Under the conditions of this experiment, cells infected with FU phage yielded $< 1$ phage per infected cell. (Infective centers were assayed after dilution in Tryptone medium; under these conditions the burst size is nearly normal (Shimura, Moses & Nathans, manuscript in preparation).) Since FU-RNA is an excellent messenger in cell extracts (Shimura, unpublished results), proteins formed on the input RNA should be made in normal amount. As shown in Fig. 6, however, there is little or no incorporation of histidine, lysine or arginine into protein after infection of actinomycin-treated cells with FU-MS2, even though the FU-RNA entered the cells, as determined by infective centers (see legend of Fig. 6). We conclude from these results also that the protein synthesis we observe after infection with normal MS2 is due to reading of newly formed RNA.

In the third experiment we examined the effect of withholding uridine from a (leaky) uridine auxotroph on the synthesis of phage-specific proteins. As shown in Fig. 7, reduced RNA synthesis was associated with decreased synthesis of histidine-containing protein and coat protein, again pointing to newly formed RNA as the template for these proteins.

† Abbreviation used: FU, 5-fluorouracil.

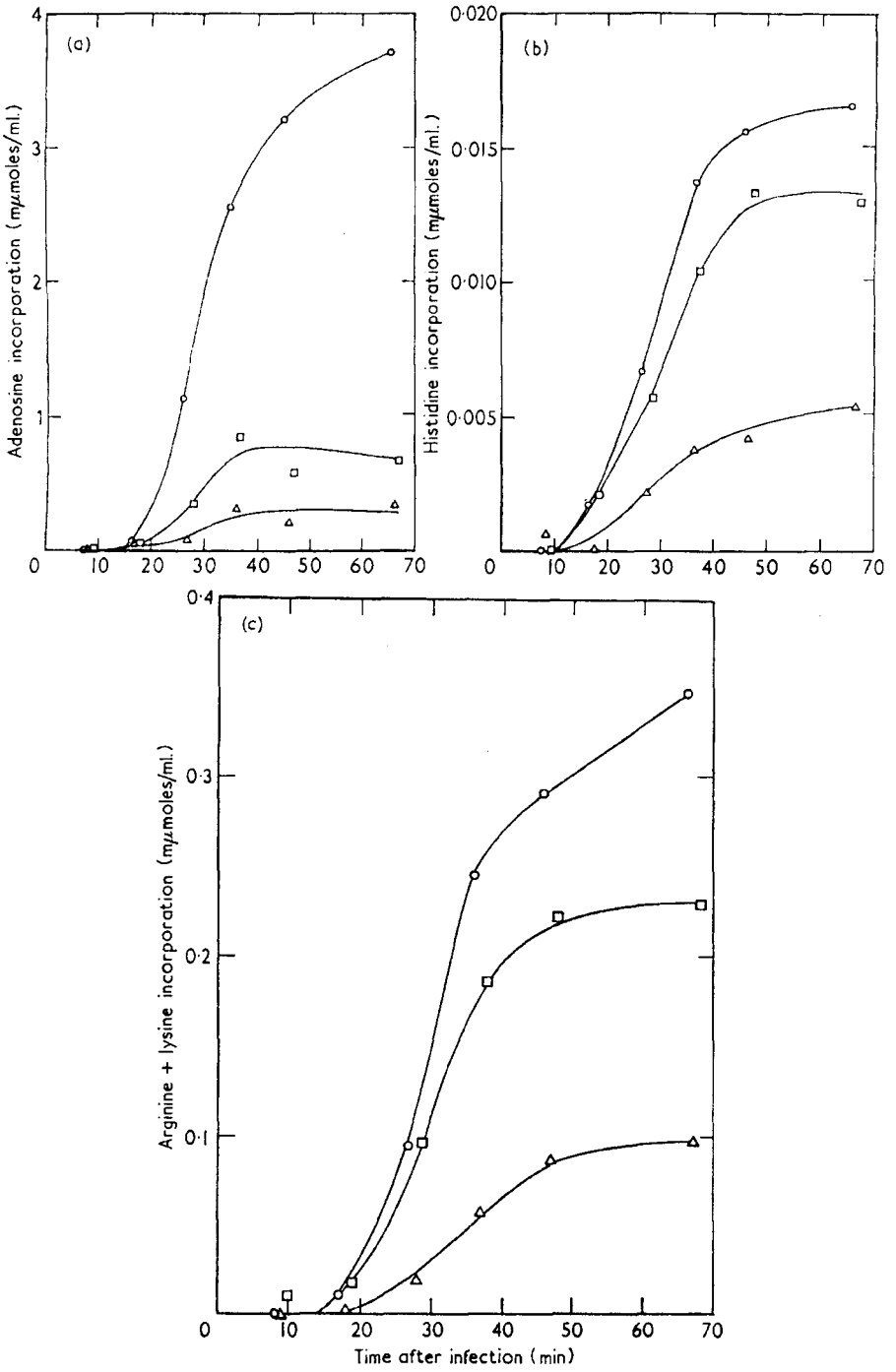


FIG. 5. The effect of 5-fluorouracil on RNA and protein synthesis.

The general procedure was that given in the legend of Fig. 2. The final concentration of histidine was $2{\cdot}8 \times 10^{-6}$ M, arginine and lysine $4{\cdot}5 \times 10^{-6}$ M each, and adenosine $3 \times 10^{-5}$ M. 5-Fluorouracil was added to one portion of culture at 5 min before infection and to a second portion at 5 min after infection, the final concentration being 60 μg/ml. Formation of infective centers (assayed after dilution in broth) was unaffected by fluorouracil addition. (○) No fluorouracil; (□) fluorouracil added 5 min after infection; (△) fluorouracil added 5 min before infection.

Finally, a temperature-sensitive mutant of MS2, defective in RNA synthesis at 42°C, was used to infect actinomycin-treated cells. As shown in Table 2, under non-permissive conditions no amino acid incorporation was detected.

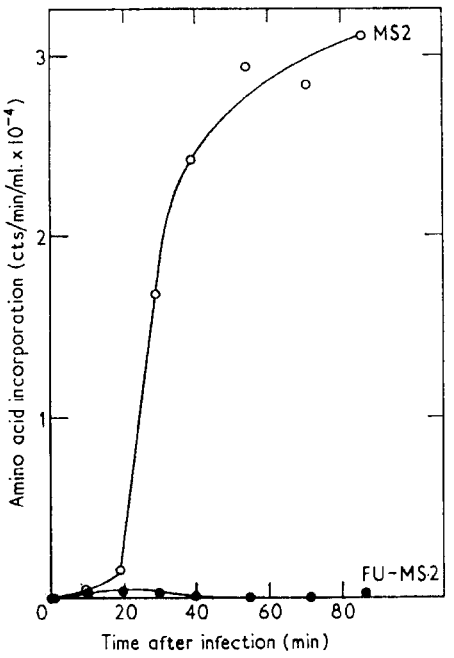


FIG. 6. Infection with fluorouracil-containing MS2.

The general procedure was the same as noted in the legend of Fig. 2 except that [$^{14}$C]arginine, [$^{14}$C]lysine and [$^{14}$C]histidine were present at the time of infection. (○) Infection with normal MS2; (●) infection with MS2 with 80% replacement of uracil by fluorouracil. Multiplicity of infection was 10. Infective centers, assayed after dilution in broth, were as follows: normal MS2, $10^7$/ml; fluorouracil phage, $9{\cdot}6 \times 10^6$/ml.

TABLE 2

*RNA and protein synthesis after infection by mutant* ts4

| Phage | cts/min incorporated per 0·1 ml. | | | | | |
|---|---|---|---|---|---|---|
| | *Uridine* | | *Histidine* | | *Lysine* | |
| | 34°C | 42°C | 34°C | 42°C | 34°C | 42°C |
| MS2 | 5000 | 6000 | 167 | 155 | 1520 | 1200 |
| *ts4* | 2900 | 250 | 100 | 0 | 720 | 0 |

Conditions were similar to those given in the legend of Fig. 2. In each case cells were infected at 34°C at a multiplicity of 10. 2 min later antiserum was added and infected cells immediately distributed to tubes with appropriate labeled precursors at 34° or 42°C. The reported cts/min are the net amounts incorporated into RNA or protein during the 60 min following infection.

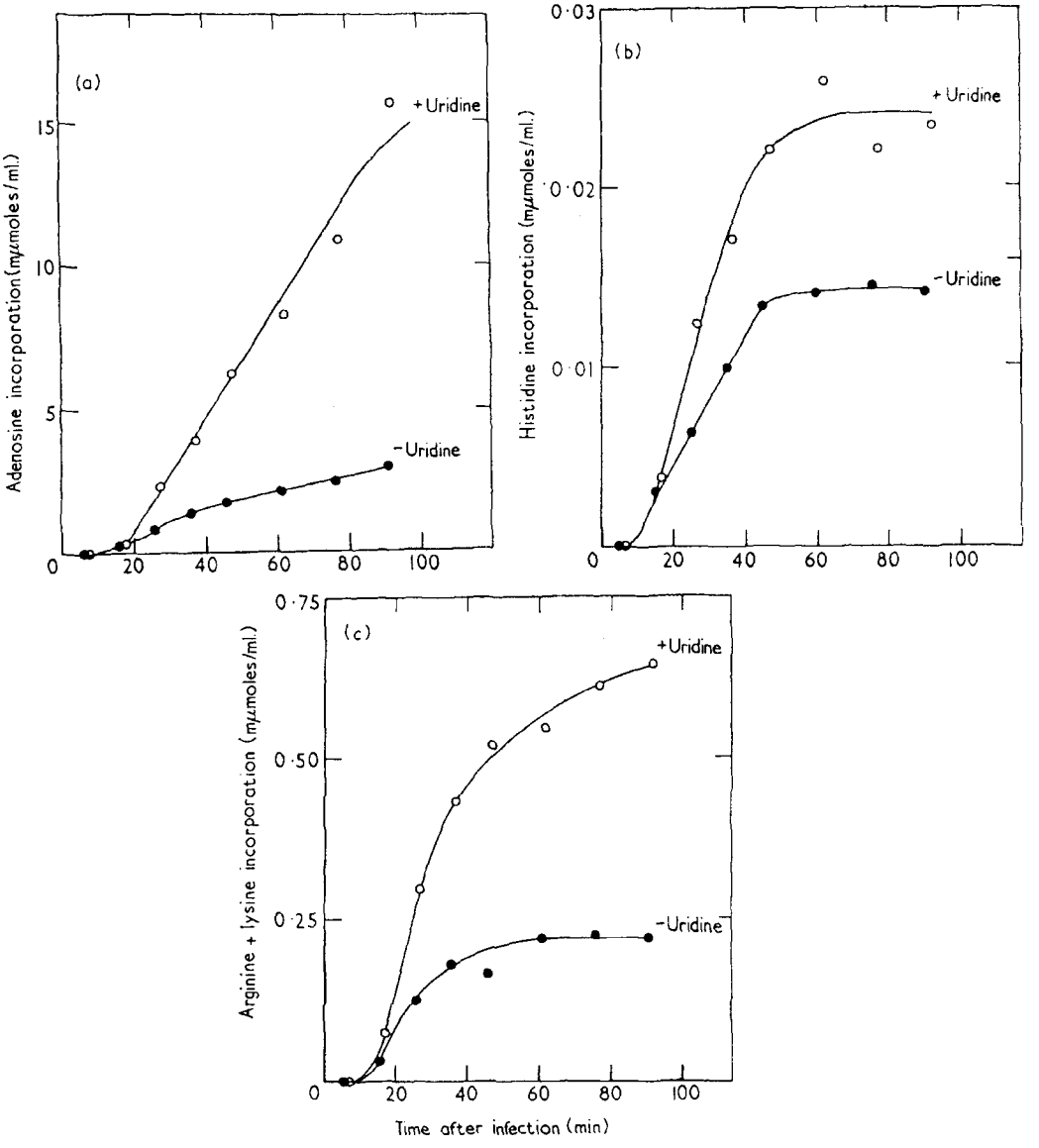


FIG. 7. Effect of uracil starvation on histidine and arginine incorporation.

Strain C3000–38, starved of uracil, was treated with 5·7 μg/ml. actinomycin as described in Materials and Methods and infected with MS2 at a multiplicity of 10. After 5 min at 37°C, 1-ml. portions were distributed to tubes containing [$^{3}$H]adenosine, [$^{14}$C]histidine or [$^{14}$C]arginine + $^{14}$C]lysine. (●) Minus uridine; (○) 20 mμmoles of uridine added 5 min after infection.

## 4. Discussion

### (a) *Effect of actinomycin on phage development*

In contrast to the lack of effect of actinomycin on the yield of most picornaviruses grown in animal cells (Reich, Franklin, Shatkin & Tatum, 1962), actinomycin has a marked effect on MS2 development in *E. coli*. Although Haywood & Sinsheimer (1963) found no fall in burst size of MS2-infected spheroplasts treated with actinomycin after infection, in our experiments, in which cells were infected after virtual cessation of protein synthesis, a fall in infective centers and in burst size was observed. We are at present unable to localize the inhibitory effect of actinomycin with any precision. Whether this effect is secondary to inhibition of host protein synthesis or to a hitherto undescribed binding of actinomycin to a molecule other than DNA is not known. In view of the low yield of MS2 in actinomycin-treated cells, and the formation of a large amount of fragmented RNA (Kelly, Gould & Sinsheimer, 1965), results obtained in this system may not be directly applicable to normal phage development. However, the observations that in the presence of actinomycin the time of formation of infectious phage is normal and the bulk of coat protein formed enters phage particles suggest that certain findings, particularly in regard to phage-specific proteins, are likely to be relevant to normal phage replication.

### (b) *"Early" protein(s)*

It has been shown in many laboratories that a virus-induced enzymic activity ("RNA synthetase") appears in cells infected with RNA phage prior to the appearance of infective particles (Weissmann, Simon & Ochoa, 1963; August, Cooper, Shapiro & Zinder, 1963; Haruna, Nozu, Ohtaka & Spiegelman, 1963), and that this activity levels off prior to completion of the replicative cycle. However, in view of the dependence of this enzyme assay on template RNA as well as newly formed protein, it was not clear that the assay actually reflected enzyme concentration rather than template concentration. By following the incorporation into protein of histidine, an amino acid lacking in phage coat, we detected "early" protein synthesis (i.e., earlier than coat protein synthesis) which follows closely the kinetics of formation of RNA synthetase activity detected by others.

Lodish *et al.* (1964) and Lodish & Zinder (1966) have presented evidence that only the input strand of RNA is used as template for the formation of "RNA synthetase". We have concluded, on the other hand, that both the "histidine protein" and coat protein are made on progeny RNA molecules, since FU added five minutes before infection or a limited supply of uridine inhibits the synthesis of "histidine-protein" and coat protein proportionately. Evidently the small amount of protein made on the input RNA (Lodish & Zinder, 1966) is not detectable by amino acid incorporation, a conclusion confirmed by our failure to find protein or RNA synthesis in actinomycin-inhibited cells infected with FU-containing MS2 or with a temperature-sensitive mutant of MS2, defective in RNA synthesis. If the histidine-protein is largely the "RNA synthetase", the difference between the results of Lodish *et al.* (1964) and those presented here may reflect an abnormality of phage development in actinomycin-treated cells.

The "early" and "late" phage-specific proteins differ in both time of formation and quantity, even though both appear to be made on newly synthesized RNA. At present we have no explanation for the sequential synthesis of these proteins, although the

similarity to the sequential synthesis of induced or de-repressed proteins from a single operon may be significant (Alpers & Tomkins, 1965; Goldberger & Berberich, 1965). Neither can we explain the reversed sequence of coat protein synthesis followed by histidine incorporation observed in *E. coli* extracts programmed with MS2 RNA (Ohtaka & Spiegelman, 1963).

The different amounts of "early" and "late" proteins synthesized have already been noted (Table 1). Considering only the time period between about 25 and 50 minutes, i.e., after the maximal rate of synthesis of late protein has been reached, we estimate that the number of coat protein molecules synthesized is at least 15 times the number of molecules of a histidine-containing protein synthesized during this same period. Since this estimate is based on the assumptions that there is only one histidine residue per protein molecule and only one histidine-protein, it is likely that the actual number of molecules of a specific histidine-containing protein made in the infected cell is still lower by several fold. Therefore, if one assumes that only whole molecules of viral RNA serve as messenger, the rate of reading of different cistrons of the polycistronic viral RNA must vary, at least during the later stages of the infective cycle. A similar conclusion was reached by Summers *et al.* (1965) in the case of poliovirus.

### (c) *Number and function of the "histidine-proteins"*

The number and functions of the "histidine-proteins" identified here are not yet known. Haywood & Sinsheimer (1965) have presented evidence for more than one "histidine-protein" and Lodish *et al.* (1965) and Heisenberg (1966) have genetic evidence for a protein which may protect viral RNA. Our experiments with FU suggest that at least part of the "histidine-protein" is concerned with RNA synthesis. It is therefore possible that the RNA synthetase recently purified by Spiegelman, Haruna, Holland, Beaudreau & Mills (1965) is present in this fraction. More precise definition of these proteins should emerge from studies with phage mutants and by fractionation of the proteins.

We are grateful to Jo Ann Fuller and Charles Gress for able technical assistance. This research has been supported by the National Institutes of Health, United States Public Health Service. One of us (M. P. O.) is a postdoctoral fellow of the U.S. Public Health Service.

*Note added in proof:* We have recently confirmed the differential rates and times of synthesis of coat and non-coat proteins in actinomycin-treated *E. coli* infected with MS2 by measurements of phage-specific proteins separated by electrophoresis in polyacrylamide gel (Nathans, D., Oeschger, M. P., Eggen, K. & Shimura, Y. (1966), *Proc. Nat. Acad. Sci., Wash.*, in the press).

After submission of this paper, two reports appeared on the inhibitory effect of actinomycin on MS2 development (Haywood, A. M. & Harris, J. M. (1966), *J. Mol. Biol.* **18**, 448; and Lunt, M. R. & Sinsheimer, R. L. (1966), *J. Mol. Biol.* **18**, 541). The results of these authors are similar to those reported above, except that under the conditions used by us, more actinomycin is required to inhibit MS2 replication as well as host RNA and protein synthesis. We assume that this difference is related to the method of sensitizing *E. coli* to actinomycin.

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